FINAL REPORT

PHASE I: DETERMINATION OF HEALTH EFFECTS OF ENVIRONMENTAL POLLUTANTS USING AVIAN MODELS: A HOLISTIC APPROACH

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A Report to
Michigan Great Lakes Protection Fund

SECTION 1.0

EXECUTIVE SUMMARY

The use of the bald eagle as an ecosystem monitor of Great Lakes Water Quality has been proposed by the International Joint Commission. Recent studies have shown that the eagle is an appropriate model for assessing the spatial and temporal trends of persistent toxic substances and has been selected by the Michigan Department of Environmental Quality as a biosentinel species. This project furthers the development of health effects biomarkers that could be coupled to the annual survey of persistent toxic compounds in bald eagle blood and feather samples.

Recently, we have found that at least one immune system biomarker, corticosterone levels, is highly correlated with either p,p'-DDE or Total PCB concentrations in blood (Bowerman *et al.* 2002). Corticosterone functions, in part, in the regulation of fat deposition and storage and in immune system functions. For migratory birds and those that exist in cold climates, alteration of fat deposition could adversely impact survival. Further, a compromised immune system may also decrease survivability. The potential to predict survivability through the use of biomarkers is an encouraging finding. Therefore, we proposed to repeat our field trials and expand the scope of our research by linking the field project to a laboratory study using chickens as a surrogate species for bald eagles.

We report here on the first year of a two-year grant looking at developing these biomarkers for the bald eagle biosentinel project. This study will attempt to describe a holistic approach to predicting impaired survivability in eagles exposed to persistent, bioaccumulative and toxic contaminants (PBTs). This assessment method will include biomarkers of the endocrine, immune, circulatory, and detoxification systems and general indicators of health including growth rates, body mass, parasitism and birth defects. These indicators will be correlated to exposure to PBTs. Relationships between health indicators and PBT contaminants will be explored using controlled laboratory tests. Those tests that prove to be predictive will be considered for incorporation into the MDEQ Strategic Monitoring Program.

We have completed a number of field derived biomarker measurements using plasma and fecal swabs from nestling bald eagles. Those that have shown significant differences among regional subpopulations include levels of Vitamin A, Vitamin E, the thyroid hormones T3 and Free T4, and possibly Con-A as an immune system endpoint. While the ACTH challenge test did not show any regional differences, based on our previous paper, it still holds some promise. All levels will be compared to concentrations of organochlorine pesticides, PCBs, and mercury to further elicit relationships and to evaluate potential biomarkers.

We have completed one of two controlled lab studies using chickens dosed *in ovo* with PCB126. We have completed a number of biomarker measurements using plasma, organs, and fecal swabs. Significant differences were observed among dose groups for immune organ weights and index values, immune system tests for PHA, and bursal cell density, the P450 related markers EROD and GST, and possibly fecal bacteria. Other markers showing some promise include Vitamin A, Vitamin E, ACTH, and the Sheep Red Blood Cell immune assay.

SECTION 2.0

INTRODUCTION

The use of the bald eagle as an ecosystem monitor of Great Lakes Water Quality has been proposed by the International Joint Commission. Recent studies have shown that the eagle is an appropriate model for assessing the spatial and temporal trends of persistent toxic substances and has been selected by the Michigan Department of Environmental Quality as a biosentinel species. This project furthers the development of health effects biomarkers that could be coupled to the annual survey of persistent toxic compounds in bald eagle blood and feather samples.

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We report here on the first year of a two-year grant looking at developing these biomarkers for the bald eagle biosentinel project. This study will attempt to describe a holistic approach to predicting impaired survivability in eagles exposed to persistent, bioaccumulative and toxic contaminants (PBTs). This assessment method will include biomarkers of the endocrine, immune, circulatory, and detoxification systems and general indicators of health including growth rates, body mass, parasitism and birth defects. These indicators will be correlated to exposure to PBTs. Relationships between health indicators and PBT contaminants will be explored using controlled laboratory tests. Those tests that prove to be predictive will be considered for incorporation into the MDEQ Strategic Monitoring Program.

SECTION 3.0

STUDY DESIGN AND METHODS

3.1 BALD EAGLES

3.1.1 *Blood/Feather Collection*

Blood was collected from nestling bald eagles in Michigan and northern Minnesota. Nestling eagles were sampled during normal banding activities. A climber would climb up the tree to the nest, then lower one nestling to the ground for blood collection. Sterile techniques were used to collect blood from the brachial vein with heparinized syringes fitted with 22 or 25 gauge needles. Samples of whole blood were transferred to heparinized vacuum tubes, kept on ice in coolers, and centrifuged within 48 hours of collection. Blood plasma was decanted and transferred to vacuum tubes and frozen (Bowerman *et al., In Press*).

3.1.2 *Growth and Age Determination*

We determined the age and sex of nestlings by measuring the eighth primary feather and foot pad of nestlings and using these measurements in mathematical growth rate and sexual dimorphism equations (Bortolotti 1984).

3.1.3 *Vitamin Levels*

The fat-soluble vitamins A and E were analyzed from plasma samples. Plasma samples were sent to Michigan State University's Animal Health Diagnostic Laboratory for vitamin analysis. Vitamins were extracted from plasma and analyzed using a high performance liquid chromatography (HPLC) system. For the vitamin A analysis, retinol, retinol palmitate, and beta-carotene levels were measured.

Vitamin D was also analyzed in the plasma samples using the DiaSorin 25-OH-D assay. This procedure measures the amount of 25-hydroxyvitamin D (25-OH-D) and other hydroxylated vitamin D metabolites in plasma to assess vitamin D sufficiency.

3.1.4 *Thyroid Hormones*

Thyroid hormones were measured in plasma samples. The thyroid hormones measured were T_3 (triiodothyronine), T_4 (thyroxine), and free T_4. All analyses were done at Michigan State University using radioimmunoassays.

3.1.5 *Corticosterone*

The ACTH stimulation test was used as a measure of the stress response. During normal stress, animals secrete ACTH (adrenal corticotropin hormone) from the pituitary gland, which in turn stimulates secretion of corticosterone. Exposure to toxicants can reduce the normal stress response, so the purpose of this test was to determine if the dose concentrations caused a reduction in plasma corticosterone levels.

Blood was collected from 19 nestling bald eagles in Michigan in 1997. Sterile techniques were used to collect up to 12 ml of blood from the brachial vein with heparinized syringes fitted with

22 or 24 gauge needles prior to administration of ACTH. Samples of whole blood were transferred to heparinized vacuum tubes (7.5 ml) or to EDTA vacuum tubes (1.5 ml), or vacuum tubes with no additive (1 ml), and kept on ice in coolers. After initial venipuncture, 0.125 mg ACTH was administered intramuscularly into the pectoral muscle. After 30 min a second venipuncture occurred and 1.5 ml of blood was collected and transferred to a second EDTA vacuum tube. The blood in the heparinized and EDTA tubes were centrifuged within 45 min of collection. Blood plasma was decanted and transferred to vacuum tubes. All vacuum tubes were then frozen. Plasma was placed in cryovials and stored in a -80°C freezer until transport to Michigan State University for analysis. Plasma corticosterone levels were analyzed by a solid-phase radioimmunoassay. ACTH response was calculated as the difference in corticosterone levels between T_0 and T_{30}.

3.1.6 *Bald Eagle Peripheral Blood Lymphocyte Mitogenesis*

Peripheral blood lymphocyte (PBL) samples were acquired from three immature captive and four wild nestling bald eagles in Spring of 2000 for evaluation of potential for use in *in vitro* mitogenesis as a biomarker of immune function. Samples were also collected from wild bald eagle nestlings in the Spring of 2001.

PBL were isolated from heparinized whole blood using a slow spin centrifugation method. The PBL buffy coat was swirled off the red blood cell pellet and removed with the plasma fraction over two 120xg spins (20 and 5 minutes). PBL were pelleted and plasma stored for other analyses. PBL were cryopreserved using standard methods and stored long-term in liquid nitrogen. Upon thaw at 37C, PBL were diluted from the freezing medium 10x using RPMI. PBL were washed and viability and cell type enumerated. Viable samples were assayed for proliferation after stimulation with T-cell mitogens PHA or Con-A, the T and B-cell mitogen PWM, the B-cell mitogen LPS and combinations of the latter three mitogens with the protein kinase-C activator, PMA. Proliferation was measured between 24 and 42 hours or 48 and 66 hours of culture using a BrdU incorporation ELISA kit (Roche Molecular). Due to small numbers of cells and samples available only a few concentrations of mitogens could be evaluated.

3.1.7 *Parasites*

Thin blood smears were prepared in the field using a drop of blood from the brachial vein. The smears were air dried immediately and stored in an envelope or slide box to protect them from insects. They were later fixed in absolute methanol and stained with Giemsa in the laboratory. Each slide was examined for parasites in its entirety using low magnification (100x) and for an additional 5 min under oil immersion (970x). Each slide where parasites were not observed was re-examined. The degree of parasitemia was estimated on a logarithmic scale of 0 to 4 following the method of Ashford *et al*. (1990) as follows: 0 = no parasites seen in the entire smear examined at low power (100x); 1 = fewer than 1 parasite per 100 high power (400x) fields; 2 = 1-10 parasites per 100 high power fields; 3 = 11-100 parasites per 100 high power fields; 4 = more than 100 parasites per 100 high power fields. Parasites were identified by comparing color, morphology and size against descriptions of known species. Blood smears with representative

infections (Accession # 88010 and 88011) were deposited at the U.S. National Parasite Collection (USDA, Beltsville, Maryland, USA).

3.2 CHICKENS – PCB 126

3.2.1 *Dosing, Hatch, and Colony Care*

White leghorn chickens eggs were dosed with four concentrations of pure PCB 126. Dosing took place over two days, 17-18 December 2001. Dosing solutions included 0.1, 0.175, 0.250, and 0.325 ng/g of PCB 126 in a sunflower oil carrier. Control and vehicle control groups were also present. A total of 248 white leghorn chicken eggs were randomized and candled to determine placement of the air cell, which was marked with a pencil. Eggs were then weighed to determine the amount of dosing solution to inject. 0.100 µl of each dosing solution per gram of egg weight was injected using Hamilton® syringes. Prior to injection, the egg shell areas above the air cells were wiped with 70% ethanol and a small hole was made using a dissection probe. The dosing solution was then injected into the air cell and the hole was covered with a thin layer of paraffin wax. Eggs were kept at room temperature until transport to the Clemson University Morgan Poultry Center on 19 December where they were placed in an incubator. Table 3.2.1.1. shows the number of eggs dosed per group.

Table 3.2.1.1. Number of chicken eggs dosed per concentration of PCB 126.

Group	**# Eggs Dosed**
Control	26
Vehicle Control (sunflower oil)	26
0.1 ng/g PCB 126	30
0.175 ng/g PCB 126	30
0.250 ng/g PCB 126	56
0.325 ng/g PCB 126	80
Total	248

Eggs remained in the incubator from 19 December 2001 until 6 January 2002 when they were transported to the hatcher. Eggs were candled on 2 January and 6 January to identify any non-viable eggs, which were removed at that time and examined for stage of death and any malformations. Eggs hatched on 9 January 2002. Hatched birds were weighed and given wing tags for identification. They were placed in a brooder and immediately given food and water. After all birds hatched they were transported to Godley-Snell Research Facility at Clemson where they were placed in a commercial brooder and separated by dose group. All eggs that did not hatch were examined for stage of death and any malformations were noted.

Food and water were given *ad libitum* and checked daily. The food was a non-medicated, standard chicken feed prepared at the Clemson University Morgan Poultry Center. Cage liners were changed daily and birds were observed for any signs of distress. Weights were taken weekly on each chick. Birds were moved to larger floor pens at four weeks of age and wood chips were provided for bedding.

Necropsies were performed at two time periods; half of the birds were euthanized at two-weeks of age and the others were euthanized at five weeks of age. Two-week necropsies took place 22-23 January and 5-week necropsies were done 12-13 February. Birds were randomized to determine which would be euthanized at two weeks. The objective was to perform necropsies on a total of 10 birds per treatment group during each time period. Lower than expected hatching success made this impossible for the control and vehicle control groups. The 0.100 ng/g group was also lower than expected because 2 birds in this group had to be euthanized post-hatch. It was determined to keep 10 birds in each group for the 5-week necropsies. 43 birds in total were culled from the two highest dose groups to reduce our experimental unit numbers to the desired 10 birds per dose group per necropsy period. However, due to counting errors, only 9 birds were left in the 0.100 ng/g group and necropsies were performed on 11 birds in the 0.250 ng/g group. See Table 3.2.1.2. for sample numbers at each necropsy.

Table 3.2.1.2. Total number of chickens necropsied per treatment level at 2 and 5 weeks of age.

Treatment	**2-week necropsy**	**5-week necropsy**	**Total**
Control	8	10	18
Vehicle Control	4	10	14
0.100 ng/g	9	9	18
0.175 ng/g	10	10	20
0.250 ng/g	10	11	21
0.325 ng/g	10	10	20
Total	51	60	**111**

Birds were euthanized using a precharged CO_2 chamber. Immediately following euthanasia, a blood sample was taken by heart stick and placed in an EDTA tube. These samples were centrifuged to separate the plasma from blood cells. 1 ml of plasma was put into each of two cryovials when total volume permitted. If volume was inadequate, one cryovial was filled to 1 ml of plasma and the remainder placed in the second vial. These samples were then placed in a -80°C freezer. Following blood collection, cloacal swabs were taken from the birds and used to inoculate agar plates. The organs were then harvested from the birds and weighed. The brain, liver, heart, right and left thymus, and bursa were harvested from all birds and gall bladders were taken from the 5-week birds. Half of the brain and the entire heart were placed in a single container of 10% buffered formalin solution. The other half of the brain and the entire liver were wrapped in foil and placed in a -80°C freezer. Gall bladders were placed in cryovials and stored in the -80°C freezer as well. The bursa and thymi were disposed of after weights were recorded.

In addition to these procedures, thymocyte and bursal cell density was calculated at the 2-week necropsy. Analysis of all immune function tests was done at Wright State University. One cryovial of plasma was sent to Michigan State University for thyroid hormone and vitamin analysis. The remaining cryovials will be sent to the University of Florida for estrogen, testosterone, and vitellogenin analysis. Intestinal bacteria and liver enzyme levels were analyzed at Clemson University.

3.2.2 *Growth and Organ Weights*

Birds were weighed at hatch and each following week until they were euthanized. Therefore, three weights were obtained for all birds and six weights were recorded for the half euthanized at 5 weeks of age. Due to higher than expected hatchability in the two highest dose groups, a group of birds was culled after two weeks and no necropsies were done. However, their weights were recorded at hatch and the first week and they are included in this analysis. All weights were recorded to one tenth of a gram. Comparisons were made between each dose group for each weight.

All organs harvested at both necropsies had weights recorded to one thousandth of a gram. These organs included the brain, liver, heart, left and right thymus, and bursa. Gall bladders were not weighed. Comparisons were made between weights for each dose group at each age.

3.2.3 *Vitamin Levels*

The fat-soluble vitamins A and E were analyzed from plasma samples taken at the 2-week and 5-week necropsies. Plasma samples were sent to Michigan State University's Animal Health Diagnostic Laboratory for vitamin analysis. Vitamins were extracted from plasma and analyzed using a high performance liquid chromatography (HPLC) system. For the vitamin A analysis, retinol, retinol palmitate, and beta-carotene levels were measured.

Vitamin D was also analyzed in the plasma samples using the DiaSorin 25-OH-D assay. This procedure measures the amount of 25-hydroxyvitamin D (25-OH-D) and other hydroxylated vitamin D metabolites in plasma to assess vitamin D sufficiency.

3.2.4 *Thyroids*

Thyroid hormones were measured in plasma taken at the 2-week and 5-week necropsies as an indicator of thyroid function. The thyroid hormones measured were T_3 (triiodothyronine), T_4 (thyroxine), and free T_4. All analyses were done at Michigan State University using radioimmunoassays.

3.2.5 *Corticosterone*

The ACTH stimulation test was used as a measure of the stress response. During normal stress, animals secrete ACTH (adrenal corticotropin hormone) from the pituitary gland, which in turn stimulates secretion of corticosterone. Exposure to toxicants can reduce the normal stress response, so the purpose of this test was to determine if the dose concentrations caused a reduction in plasma corticosterone levels.

The test was performed on five birds in each dose group (4 in the 0.100 ng/g group). An initial blood sample (T_0) was obtained from the jugular vein of each bird at 29 days of age. Because limited blood volume can be taken from birds of this size at any one time, the ACTH injection was not done until 33 days of age. At this time, 2.5 IU Cortosyn® was injected into the breast muscle and a second blood sample was obtained 30 minutes post-injection (T_{30}). In both cases, blood samples were immediately placed in EDTA tubes and centrifuged for plasma separation. Plasma was placed in cryovials and stored in a -80°C freezer until transport to Michigan State

University for analysis. Plasma corticosterone levels were analyzed by a solid-phase radioimmunoassay. ACTH response was calculated as the difference in corticosterone levels between T_0 and T_{30}.

3.2.6 *Immunotoxicology*

Several immune function tests were performed on the hatched birds, including the phytohemaglutinnin (PHA) skin response test, the sheep red blood cell (SRBC) hemagglutination assay, and measures of immune organ mass and cell density.

The PHA skin test is an *in vivo* immune function test measuring a T-cell mediated inflammatory response and is similar to delayed type hypersensitivity responses. It involves a polyclonal T-cell mitogen as an antigen rather than a specific antigen that would require testing of a memory response. 15 birds in each dose group (14 in vehicle control) received this test at 11 days of age. Each chick was given an interdigital injection of 0.03 ml of PHA solution (containing 100 μg of PHA) into one foot web. The other foot web received a 0.03 ml injection of sterile PBS. Both foot webs were measured prior to injection and 24 hours after injection. The mitogen simulation index was calculated as the increase in thickness caused by PHA minus the increase caused by PBS.

The hemagglutination assay measures the antibody response after injection of an antigenic foreign substance, in this case, sheep red blood cells. A 1% suspension of SRBCs was prepared when the birds were 22 days old. Five birds in each dose group received a 0.1 ml injection of this suspension via the jugular vein. Blood was collected for analysis 6 days post injection, which is when peak antibody activity usually occurs. Collected blood was centrifuged and plasma was stored in a -80°C freezer until transport to Wright State University for analysis. Plasma antibody activity was then measured by a microtiter procedure.

Several measurements were taken from the euthanized birds. At two and five weeks of age, immune organs were weighed and the thymus index and bursa index were calculated (organ mass divided by body mass). At two weeks of age, live thymocytes in the left thymus were counted and the thymocyte density was calculated (number of live thymocytes divided by the organ mass). Live bursal cells were also counted at two weeks and the bursal cell density was calculated.

Data were analyzed using the nonparametric Jonckheere test, an *a priori* test that detects monotonic but not necessarily linear dose responses.

3.2.7 *Cytochrome P450/Phase 2 Enzymes*

Depending on the chlorine substitution pattern, PCBs are more or less easily oxygenated by cytochromes P450. PCB126 (3,3’,4,4’,5-pentachlorobiphenyl) has two neighboring unsubstituted carbon atoms on one of the phenyl rings, and therefore is a potential substrate for cytochrome P-450 oxygenation. Trough a 1,2-shift 4-hydroxy-,3,3’,4’,5,5’-pentachlorobiphenyl can be formed.

OH-PCBs like this, with the hydroxyl group in the para position, showed to be very potent inhibitors of phase 2 enzymes when tested in vitro.

Most metabolized PCBs are excreted through urine and bile. Some hydroxylated PCB metabolites are found to accumulate in blood plasma. These metabolites have a chlorine substitution pattern that resembles the iodine substitution on thyroxine, a thyroid hormone, which enables them to bind to the thyroxine transport protein. This apparently causes reduced blood plasma thyroid levels, which has been linked to impaired fetal development. PCBs can also induce UDP-glucuronosyltransferase (UGT), including the form that glucuronidates thyroxine. Several OH-PCBs have been described as inhibitors of thyroxine sulfation, the major regulatory pathway for this thyroid hormone. More recently, several OH-PCBs were found to be very potent inhibitors of estrogen sulfotransferase, which may explain the estrogenic effect of PCBs.

In the current project chicken eggs were injected with PCB126. This opened the opportunity to study in vivo how phase 1 and phase 2 enzymes are affected by PCB126 and its hydroxylated metabolites. Planar PCBs are well know inducers of CYP1A through activation of the AH receptor. CYP1A induction was measured with the EROD assay. Of the phase 2 enzymes, glutathione-S-transferase (GST) and uridine-diphospho- glucuronosyltransferase (UGT) are known to be induced by AH receptor agonists, while sulfotransferases (SULT) are generally not inducible. OH-PCBs are inhibitors of UGT and SULT, and inhibition of these conjugating enzymes might be compensated for by increasing the total enzyme amount. The catalytic activity of the three phase 2 enzymes was measured, and the total enzyme amount will be measured to see if inhibition was compensated by increased enzyme concentrations.

As of now only samples from the two week old chickens have been processed for the enzyme studies. For each enzyme assay five replicates per treatment group were analyzed, except for the vehicle control, where due to lack of samples, only 4 replicates could be analyzed.

Liver samples were kept at -80º C until use. Microsomes and cytosol were prepared by differential centrifugation and were stored at -80º C in buffered media with 20 % glycerine. Protein content of the samples was measured with the bicinchoninic colorimetric assay (Pierce).

Cytochrome P450-1A activity was measured with ethoxyresorufin as a substrate. Microsomes (100 μg/ml) were incubated at room temperature and fluorescence of the increasing product resorufin was measured over 30 min in a 96-well plate reader.

GST activity was measured as the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB) by cytosolic protein. Increasing absorption of the conjugated product was measured over a two minute period in a spectrophotometer, using the kinetics option to calculate the rate of conversion.

UGT and SULT activities were measured with 9-hydroxy-benzo[a]pyrene as a substrate for the conjugation reactions. For SULT activity 25 μg cytosolic protein was incubated for 10 min with the substrate and adenosine-3',5'-diphosphosulfate (PAPS) as sulfate donor. For UGT 50 μg microsomal protein was incubated with the substrate and UDP-glucuronic acid as co-substrate.

After increasing the alkalinity till pH 11, the conjugated product was measured at the specific wavelength pairs in a fluorescence plate reader.

Significant differences ($p<0.05$) between treatment groups were detected using one-way ANOVA, followed by Tukey's multiple comparison test.

3.2.8 *Intestinal Bacteria*

Intestinal bacteria were examined for differences in types of bacteria among the doses and antibiotic susceptibility profiles. The hypothesis was that reduced immune function in dosed birds would lead to the colonization of different and possibly more pathogenic strains of bacteria in the intestinal tract.

At dosing, a group of eggs was tested for bacteria to obtain baseline data. Intestinal flora of juvenile chickens was collected by cloacal swab at two-weeks and five-weeks of age, following euthanasia. The swabs were used to inoculate bacterial cultures using both gram-positive and gram-negative media. The three predominant aerobic bacteria were identified by colony observation and tested for each bird. Two gram-negative bacterial types and one gram-positive bacterial type were used, as these were the most frequently observed and consistent colonies among the samples. Colony identification and antibiotic susceptibility profiles were tested using MicroScan® panels Positive Combo 14 and Negative Combo 22. Quality control organisms were tested along with the samples.

Each bacteria identified was tested for antibiotic susceptibility by observing the presence or absence of growth in varying concentrations of antibiotics. Gram-negative bacteria were grown in the presence of 24 different antibiotics; gram-positive bacteria were grown with 22 antibiotics. Bacteria were determined to be either susceptible or intermediate in resistance to each antibiotic, using criteria from the NCCLS (National Committee for Clinical Laboratory Standards) Document M100-S7, as reported in the MicroScan Procedural Manual. Complete resistance could not be determined with the test system that was used.

Results for microbial identification and antibiotic susceptibility profiles were analyzed using a chi-square test and the Pearson chi-square statistic was used to determine significance

SECTION 4.0

RESULTS AND DISCUSSION

4.1 BALD EAGLES

4.1.1 *Vitamin Levels*

Vitamin data were analyzed by region in two ways. The first analysis compares data between five regions: Lake Superior (LS), Inland MI (I-MI), Voyageurs National Park (VNP), Lake Michigan/Huron/Erie (LMHE), and Saginaw Bay (SB). The second analysis groups the data as either Great Lakes (LS, LMHE, SB) or Inland (I-MI, VNP) for comparison.

In the first analysis, significant differences were found between regions for Vitamin E (χ^2=18.7951, df=4, p=0.0009), retinol (χ^2=22.0886, df=4, p=0.0002), retinol palmitate (χ^2=12.8538, df=4, p=0.0120), and beta-carotene (χ^2=9.0874, df=4, p=0.0590). Levels of vitamin E decreased from LS>LMHE>VNP>I-MI>SB. Retinol levels decreased from LS>I-MI>SB>LMHE>VNP. Retinol palmitate levels decreased from LS>VNP>I-MI>SB>LMHE. Beta-carotene levels decreased from I-MI>SB>LS>LMHE>VNP. Mean levels of vitamins E, retinol, retinol palmitate and beta-carotene are shown in Table 4.1.1.1.

Table 4.1.1.1. Mean levels of vitamin E (alpha tocopherol), retinol, retinol palmitate, and beta-carotene by region.

Region	Vitamin E (μg/ml)	Retinol (ng/ml)	Retinol Palmitate (ng/ml)	Beta-carotene (μg/ml)
Lake Superior	42.03 ± 8.50 A*	1728.42 ± 94.90 A	27370.63 ± 2282.16 A	0.15 ± 0.04 C
Inland MI	31.21 ± 5.61 B	1332.78 ± 55.48 B	12566.44 ± 12612.12 B	0.25 ± 0.24 A
Voyageurs NP	33.48 ± 5.06 B	859.47 ± 420.17 D	12611.93 ± 11110.04 B	0.12 ± 0.06 C
Michigan/Huron/Erie	41.10 ± 9.17 A	1112.22 ± 522.34 C	7866.22 ± 5338.49 C	0.14 ± 0.04 C
Saginaw Bay	30.53 ± 4.34 B	1152.75 ± 244.04 C	8340.50 ± 5031.29 C	0.20 ± 0.07 B

*Means not sharing the same letter are significantly different.

In the second analysis, Great Lakes v. Inland, there were significant differences in vitamin E (Z=-2.5105, p=0.0060) and retinol (Z=-2.3499, p=0.0094), with the Great Lakes samples having higher mean vitamin levels. There were no significant differences in retinol palmitate (Z=-0.3302, p=0.3706) or beta-carotene (Z=-0.8489, p=0.1980). Mean levels of vitamin E, retinol, retinol palmitate, and beta-carotene are shown in Table 4.1.1.2.

Table 4.1.1.2. Mean levels of vitamin E (alpha tocopherol), retinol, retinol palmitate, and beta-carotene by region: Great Lakes v. Inland.

Region	Vitamin E (μg/ml)	Retinol (ng/ml)	Retinol Palmitate (ng/ml)	Beta-carotene (μg/ml)
Great Lakes	38.57 ± 9.10	1378.38 ± 465.66	14259.40 ± 15684.40	0.15 ± 0.05
Inland	32.63 ± 5.27	1036.96 ± 484.16	12594.13 ± 11435.92	0.17 ± 0.16

4.1.2 *Thyroid Hormones*

Thyroid hormone data were analyzed by region in two ways. The first analysis compares data between five regions: Lake Superior, Inland MI, Voyageurs National Park, Lake Michigan/Huron/Erie, and Saginaw Bay. The second analysis groups the data as either Great Lakes or Inland for comparison.

In the first analysis, significant differences were found between region and T3 (χ^2=13.4239, df=4, p=0.0094) and Free T4 (χ^2=11.2222, df=4, p=0.0242). For T3 levels, concentrations decreased from LS>LMHE>I-MI>SB>VNP. Free T4 levels decreased from VNP>LMHE>SB>I-MI>LS. No significant difference was found among region for T4 levels (χ^2=5.0561, df=4, p=0.2816). Mean levels of thyroid hormones are shown in Table 4.1.2.1.

Table 4.1.2.1. Mean thyroid hormone levels by region.

Region	T4 (nmol/l)	T3 (nmol/l)	Free T4 (pmol/l)
Lake Superior	12.77 ± 4.23	3.89 ± 0.60 A*	31.54 ± 5.88 C
Inland MI	13.22 ± 3.27	3.29 ± 0.68 B	34.56 ± 7.02 C
Voyageurs NP	15.06 ± 5.26	2.96 ± 0.50 C	40.38 ± 5.18 A
Michigan/Huron/Erie	15.67 ± 5.61	3.32 ± 0.67 B	37.33 ± 10.64 B
Saginaw Bay	18.00 ± 5.39	3.24 ± 0.51 B	35.29 ± 17.32 C

*Means not sharing the same letter are significantly different.

In the second analysis, Great Lakes v. Inland, significant differences were again found in T3 (Z=-2.8338, p=0.0023) and Free T4 (Z=1.8853, p=0.0297), with Great Lakes samples having higher T3 levels and Inland Sites having higher Free T4 levels. No significant difference was found in T4 levels (Z=-0.3652, p=0.3575). Mean thyroid hormone levels are shown in Table 4.1.2.2.

Table 4.1.2.2. Mean thyroid hormone levels by region: Great Lakes v. Inland.

Region	T4 (nmol/l)	T3 (nmol/l)	Free T4 (pmol/l)
Great Lakes	14.93 ± 5.25	3.56 ± 0.66	34.24 ± 10.87
Inland	14.40 ± 4.65	3.08 ± 0.58	38.28 ± 6.43

4.1.3 *Corticosterone*

ACTH data was analyzed by region in two ways. The first analysis compares data between five regions: Lake Superior, Inland MI, Voyageurs National Park, Lake Michigan/Huron/Erie, and Saginaw Bay. The second analysis groups the data as either Great Lakes or Inland for comparison.

There were no significant differences in ACTH response among region for the first analysis (χ^2=6.0600, df=4, p=0.1947), or the Great Lakes v. Inland analysis (Z=-1.1392, p=0.1273).
Mean ACTH response, measured as the difference in corticosterone levels between T_0 and $T_{30,}$ by region is shown in Table 4.1.3.1.

Table 4.1.3.1. Mean ACTH response by region, measured as the difference in corticosterone levels between T_0 and $T_{30.}$

Region	ACTH response (ng/ml)
Lake Superior	31.50 ± 2.12
Inland MI	12.43 ± 20.34
Voyageurs NP	39.38 ± 13.54
Lakes Michigan/Huron/Erie	38.50 ± 26.73
Saginaw Bay	32.75 ± 36.28
Great Lakes	35.17 ± 29.09
Inland	26.80 ± 21.51

4.1.4 *Immunology*

Samples from the Spring 2000 collection had a post-thaw viability of 69-86% and sufficient numbers of cells for assay were acquired (between 2 and $6x10^6$ lymphocytes per milliliter of blood). Responses to mitogens were generally low but two birds responded well to Con-A stimulation (0.5 ug/well in a 66 hour assay), similar in response to frozen chicken PBL. PHA, PWM, LPS or PMA produced only negligible proliferation and combinations of mitogens with PMA did not improve proliferation. Con-A tends to be a better stimulator of lymphocytes from young animals than the other mitogens tested. From this work, there seemed to be potential for this assay for bald eagles. Whole blood mitogenesis assays by Redig et al. 1984 revealed similar results: Con-A was a better mitogen for PBL stimulation than PHA and a 48-72 hour measurement was optimal.

Only four samples have been thawed from the 2001 collection. Unfortunately none of these samples were viable, possibly because of an error in sample storage. Some samples, which were stored appropriately, have not been thawed and will be analyzed in the future.

4.1.5 *Parasites*

No significant differences were noted for *Leucocytozoan toddi* among subpopulations. The extremely dry conditions experienced during the 2001 field season and the almost non-existent populations of biting blackflies and mosquitos, both potential vectors of this pathogen, are believed to be the reason for the lack of infection of nestling eagles. This is quite different than our previous study (Stuht *et al.* 1999).

4.2 CHICKENS – PCB 126

4.2.1 *Dosing, Hatch, and Colony Care*

There were significant differences in observed versus expected hatchability. Numbers of observed and expected birds are shown in Table 4.2.1.1.

Table 4.2.1.1. Hatch numbers for PCB 126 dosing.

Group	Alive Observed	Dead Observed	Alive Expected	Dead Expected	Z obs value
Control	18	8	20	6	-4.75
Vehicle Control	14	12	20	6	-14.24
0.1 ng/g	20	10	20	10	0
0.175 ng/g	20	10	20	10	0
0.250 ng/g	43	14	20	38	50.49
0.325ng/g	39	40	20	60	46.19
Total	154	94	120	130	

The control and vehicle control groups contained lower than expected numbers and the two highest dose groups contained greater than expected numbers. The two lower dose groups were not different from their expected counts. Z obs values were calculated to determine significance. Significant differences were noted between observed and expected numbers for every group except 0.1 ng/g and 0.175 ng/g (Z crit= ±1.96).

Individual doser mortality could not be determined because each person did not dose the same number of eggs.

Several deformities were noted in the egg break-out analysis. Types of deformities included edema, gastroschisis, beak and limb malformations, and small body size. Incidence of deformity per dose group is shown in Table 4.2.1.2. Overall incidence of deformity was approximately 10% (25/248). The highest rate of deformity occurred in the 0.325 ng/g dose group (16.3%).

Table 4.2.1.2. Deformities per dose group.

Group	# of deformities	% of deformities
Control	0	0
Vehicle control	2	7.7
0.1 ng/g	2	6.7
0.175 ng/g	4	13.3
0.250 ng/g	4	7.1
0.325 ng/g	13	16.3
Total	25	10.1

Necropsies were completed successfully and samples were sent to the appropriate laboratories.

4.2.2 *Growth and Organ Weights*

No significant differences were found between dose groups for weight 1 (hatch weight) (χ^2=2.1668, df=5, p=0.8256), weight 3 (χ^2=7.3701, df=5, p=0.1945), weight 4 (χ^2=4.1807, df=5, p=0.5237), weight 5 (χ^2=3.4735, df=5, p=0.6274), or weight 6 (χ^2=2.9731, df=5, p=0.7041). Significant differences were found among dose groups for weight 2 (week 1) (χ^2=37.7855, df=5, p=<.0001). Mean weights for each dose group are shown in Table 4.2.2.1.

Table 4.2.2.1. Mean weekly growth weights of chickens.

Dose Group	Weight 1 (Hatch) (g)	Weight 2 (Week 1) (g)	Weight 3 (Week 2) (g)	Weight 4 (Week 3) (g)	Weight 5 (Week 4) (g)	Weight 6 (Week 5) (g)
Control	43.76 ± 3.26	59.95 ± 5.93	106.92 ± 15.40	178.46 ± 18.35	231.55 ± 21.78	295.34 ± 32.67
Vehicle Control	43.40 ± 3.03	59.11 ± 8.01	108.44 ± 19.45	176.30 ± 32.63	232.68 ± 42.67	291.13 ± 54.44
0.100 ng/g	45.17 ± 4.00	61.27 ± 6.42	109.28 ± 13.14	186.42 ± 25.45	251.30 ± 36.01	309.63 ± 45.08
0.175 ng/g	44.31 ± 2.12	59.36 ± 6.15	105.28 ± 15.30	164.81 ± 25.74	228.09 ± 26.43	287.74 ± 38.14
0.250 ng/g	44.44 ± 3.11	52.63 ± 4.96	98.89 ± 15.72	167.97 ± 25.59	225.52 ± 33.94	293.61 ± 43.61
0.325 ng/g	44.13 ± 2.88	53.97 ± 6.19	102.26 ± 16.10	175.46 ± 26.65	238.50 ± 36.91	314.19 ± 44.12

PCB 126 did not seem to have an effect on hatch weight. However, the two high dose groups exhibited lower mean weights than the other groups at 1 week of age. It appears that the contaminants did have a negative effect on weight gain during this time. The absence of significant difference in weights after this point suggests that the effect was not permanent.

Organs were taken from each necropsied bird. No significant differences were noted between dose groups for brain (χ^2=4.7206, df=5, p=0.4509), heart (χ^2=3.7790, df=5, p=0.5816), or liver (χ^2=4.5982, df=5, p=0.4669) weights in the two week old birds. Significant differences were noted between dose groups for left thymus (χ^2=11.0638, df=5, p=0.0501), right thymus (χ^2= 14.7016, df=5, p=0.0117), and bursa (χ^2=12.0485, df=5, p=0.0341). For each of these organs, the two high dose groups had significantly lower mean weights. No significant differences were noted between dose groups for brain (χ^2=4.6817, df=5, p=0.4559), heart (χ^2=5.0993, df=5, p=0.4039), liver (χ^2=2.6522, df=5, p=0.7534), left thymus (χ^2=1.5807, df=5, p=0.9036), right thymus (χ^2=1.9380, df=5, p=0.8577), or bursa (χ^2=3.4715, df=5, p=0.6277) weights for the five week old birds. Mean organ weights are shown in Table 4.2.2.2. and illustrated in Figures 4.2.2.1. and 4.2.2.2.

Brain, liver, and heart weights do not seem to be affected by PCB 126 concentrations. Immune organ weights show some significant differences at 2 weeks of age, but not at 5 weeks of age. The 2-week thymus and bursa weights were lower in the dosed birds, suggesting a negative effect on the development of the immune system. The absence of these effects at 5-weeks suggests that the slow growth is not permanent.

Overall, the growth and organ weights suggest that the PCB 126 concentrations may have some effect early on but are not a factor in older birds.

Table 4.2.2.2. Mean organ weights of chickens at two and five weeks of age.

Dose Group	Brain (g)	Heart (g)	Liver (g)	Left Thymus (g)	Right Thymus (g)	Bursa (g)
2 weeks						
Control	1.35 ± 0.06	0.67 ± 0.13	3.83 ± 0.90	0.32 ± 0.10	0.30 ± 0.12	0.40 ± 0.10
Vehicle	1.32 ± 0.05	0.72 ± 0.10	3.79 ± 0.35	0.27 ± 0.07	0.23 ± 0.05	0.38 ± 0.04
0.100 ng/g	1.28 ± 0.12	0.76 ± 0.12	3.74 ± 0.45	0.27 ± 0.04	0.25 ± 0.05	0.38 ± 0.07
0.175 ng/g	1.28 ± 0.09	0.69 ± 0.10	3.98 ± 0.74	0.27 ± 0.09	0.27 ± 0.08	0.36 ± 0.08
0.250 ng/g	1.32 ± 0.10	0.67 ± 0.18	3.53 ± 0.68	0.21 ± 0.08	0.18 ± 0.05	0.30 ± 0.06
0.325 ng/g	1.32 ± 0.08	0.73 ± 0.17	3.57 ± 0.90	0.21 ± 0.07	0.20 ± 0.07	0.29 ± 0.10
5 weeks						
Control	2.11 ± 0.12	2.06 ± 0.35	9.06 ± 1.00	0.92 ± 0.18	0.92 ± 0.18	1.38 ± 0.35
Vehicle	2.12 ± 0.13	2.12 ± 0.57	8.44 ± 1.60	0.92 ± 0.31	0.86 ± 0.27	1.39 ± 0.36
0.100 ng/g	2.03 ± 0.28	2.44 ± 0.29	9.68 ± 1.53	1.00 ± 0.22	0.93 ± 0.26	1.58 ± 0.44
0.175 ng/g	1.98 ± 0.22	2.25 ± 0.36	9.07 ± 1.33	0.92 ± 0.16	0.84 ± 0.17	1.32 ± 0.23
0.250 ng/g	2.06 ± 0.19	2.27 ± 0.30	8.93 ± 1.51	0.98 ± 0.18	0.94 ± 0.21	1.51 ± 0.45
0.325 ng/g	2.01 ± 0.19	2.23 ± 0.39	8.40 ± 3.27	0.94 ± 0.29	0.99 ± 0.30	1.27 ± 0.42

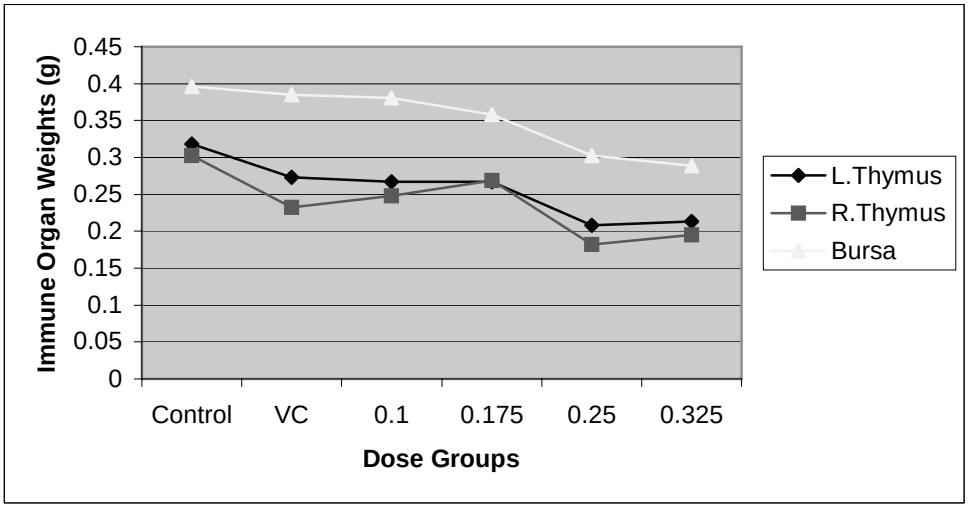

Figure 4.2.2.1. Mean immune organ weights per dose group at 2 weeks of age.

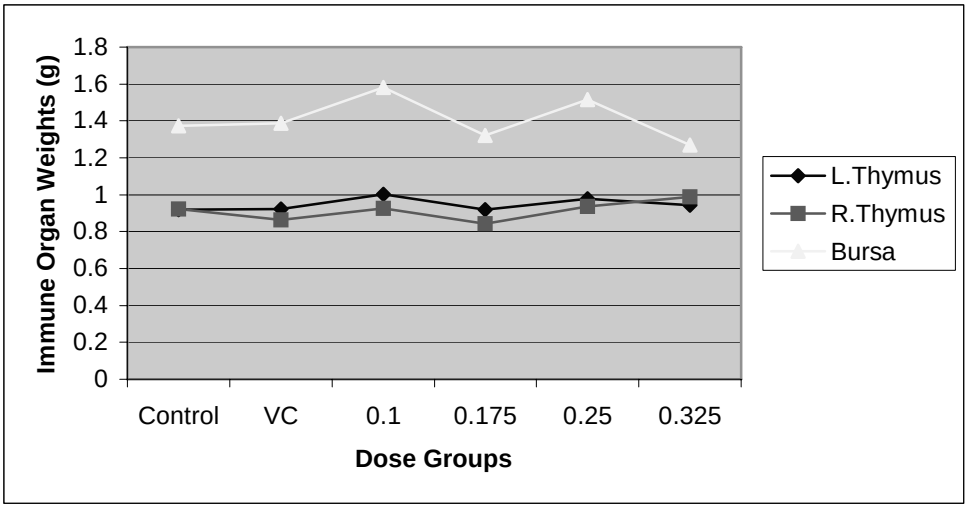

Figure 4.2.2.2. Mean immune organ weights per dose group at 5 weeks of age.

4.2.3 *Vitamins*

In the analysis of vitamin A and E, several samples had insufficient quantities of plasma available to test. Some samples within the dose groups were combined in order to obtain desired quantities. Plasma drawn for the ACTH tests (both the T_0 and T_{30} samples) were also tested for these vitamins and in some cases were combined with the 5-week necropsy plasma samples to obtain desired quantities. Therefore, some samples tested contained plasma from two or three different time periods, and the results were analyzed and are reported here in several ways. The plasma samples from the 2-week necropsies are reported separately. For the samples taken from the older birds (ACTH T_0, T_{30}, and 5-week necropsies), results are reported in three ways. First, the samples containing only 5-week necropsy plasma were analyzed separately. Second, the 5-week necropsy samples were analyzed along with plasma taken at ACTH T_0 and $T_{30,}$ but no combinations of these three times were included. Finally, all of the samples containing plasma from the ACTH T_0, T_{30}, or 5-week necropsies, or any combination of these three, were analyzed.

No significant differences were noted in either vitamin E (χ^2=1.5229, df=5, p=0.9104) or retinol (χ^2=4.1830, df=5, p=0.5234) for the 2-week samples. Mean vitamin levels are shown in Table 4.2.3.1. Significant differences were found in vitamin E levels for the 5-week sample analysis (χ^2=11.3134, df=5, p=0.0455), the 5-week/T_0/T_{30} sample analysis (χ^2=12.0647, df=5, p=0.0339), and the 5-week/ T_0/T_{30} combination sample analysis (χ^2=10.9373, df=5, p=0.0526). Significant differences were found in retinol levels for the 5-week/T_0/T_{30} sample analysis (χ^2=11.0900, df=5, p=0.0496) and the 5-week/ T_0/T_{30} combination sample analysis (χ^2=10.9779, df=5, p=0.0518), but not the 5-week only sample analysis (χ^2=9.3917, df=5, p=0.0944). The differences in retinol and vitamin E do not appear to be dose related. Mean vitamin levels for these samples are shown in Table 4.2.3.2. Retinol palmitate and beta-carotene levels were also measured but no detectable amounts were found in any birds.

Table 4.2.3.1. Mean levels of retinol, vitamin E, and vitamin D in chicken plasma at two weeks of age.

Dose Group	Retinol (ng/ml)	Vitamin E (μg/ml)	Vitamin D (nmol/l)
Control	209.00	14.29	17.33 ± 2.31
Vehicle Control	233.00	12.43 ± 5.08	16.50 ± 2.38
0.100 ng/g	322.50 ± 31.82	14.42 ± 3.87	14.44 ± 3.64
0.175 ng/g	375.33 ± 140.54	15.41 ± 2.68	17.33 ± 6.89
0.250 ng/g	369.50 ± 126.50	13.80 ± 2.12	13.10 ± 5.15
0.325 ng/g	259.00 ± 89.82	13.09 ± 1.71	± 3.93

Table 4.2.3.2. Mean levels of retinol, vitamin E, and vitamin D in chicken plasma at five weeks of age, ACTH T_0, and ACTH T_{30}.

Dose Group	Retinol (ng/ml)	Vitamin E (μg/ml)	Vitamin D (nmol/l)
5-week analysis			
Control	257.00 ± 106.07	12.59 ± 0.98	12.60 ± 3.69
Vehicle Control	226.25 ± 53.60	14.17 ± 1.65	16.50 ± 3.47
0.100 ng/g	356.67 ± 61.73	14.03 ± 2.74	14.44 ± 4.42
0.175 ng/g	196.75 ± 79.91	10.85 ± 0.32	12.90 ± 3.54
0.250 ng/g	294.88 ± 111.74	11.82 ± 1.73	15.00 ± 4.05
0.325 ng/g	345.00 ± 24.04	12.18 ± 1.84	16.80 ± 4.05
5-week/T_0/T_{30}			
Control	257.00 ± 106.07	12.59 ± 0.98	N/A
Vehicle Control	209.57 ± 49.30	13.92 ± 1.29	
0.100 ng/g	319.88 ± 87.34	13.89 ± 2.44	
0.175 ng/g	217.63 ± 71.30	11.37 ± 1.07	
0.250 ng/g	288.75 ± 100.32	12.25 ± 1.92	
0.325 ng/g	352.40 ± 83.18	12.19 ± 1.75	
5-week/T_0/T_{30} plus combinations			
Control	226.33 ± 84.35	13.16 ± 2.43	N/A
Vehicle Control	216.91 ± 84.41	13.68 ± 1.27	
0.100 ng/g	305.67 ± 92.15	13.63 ± 2.47	
0.175 ng/g	231.78 ± 79.06	11.42 ± 1.03	
0.250 ng/g	288.75 ± 100.32	12.45 ± 1.90	
0.325 ng/g	339.29 ± 74.39	20.40 ± 25.32	

There were no significant differences in vitamin D levels between dose groups for either the 2-week samples (χ^2=5.0736, df=5, p=0.4070) or the 5-week samples (χ^2=8.2863, df=5, p=0.1411). The mean vitamin D levels per dose group are shown in Tables 4.2.3.1. and 4.2.3.2.

Overall, the results suggest that vitamin A, E, and D levels are not useful biomarkers of PCB toxicity.

4.2.4 *Thyroid Hormones*

No significant differences were found among dose groups at 2-weeks for T4 (χ^2=8.7152, df=5, p=0.1210), T3 (χ^2=5.2019, df=5, p=0.3917), or Free T4 (χ^2=8.7260, df=5, p=0.1205). No significant differences were found among dose groups at 5-weeks for T4 (χ^2=2.3456, df=5, p=0.7996), T3 (χ^2=5.3491, df=5, p=0.3748), or Free T4 (χ^2=4.2912, df=5, p=0.5083). Means for each group are shown in Table 4.2.4.1 and Table 4.2.4.2. These results suggest that thyroid hormones are not a useful biomarker of PCB toxicity.

Table 4.2.4.1. Mean levels of T4, T3, and Free T4 in chicken plasma at two weeks of age.

Dose Group	T4 (nmol/l)	T3 (nmol/l)	Free T4 (pmol/l)
Control	28.00 ± 7.35	4.47 ± 0.52	15.00 ± 7.25
Vehicle Control	21.00 ± 4.40	4.50 ± 0.94	9.75 ± 1.89
0.100 ng/g	22.56 ± 7.20	4.70 ± 0.39	6.11 ± 4.31
0.175 ng/g	24.67 ± 3.50	4.46 ± 1.40	11.10 ± 6.85
0.250 ng/g	24.80 ± 3.05	4.74 ± 1.05	9.90 ± 3.51
0.325 ng/g	21.40 ± 2.91	3.78 ± 1.17	10.50 ± 4.14

Table 4.2.4.2. Mean levels of T4, T3, and Free T4 in chicken plasma at five weeks of age.

Dose Group	T4 (nmol/l)	T3 (nmol/l)	Free T4 (pmol/l)
Control	23.80 ± 6.34	5.41 ± 0.47	15.80 ± 7.02
Vehicle Control	21.60 ± 2.60	5.12 ± 1.10	10.30 ± 5.08
0.100 ng/g	20.70 ± 4.76	5.06 ± 1.27	10.80 ± 3.08
0.175 ng/g	21.30 ± 5.50	5.34 ± 1.24	10.90 ± 5.95
0.250 ng/g	22.18 ± 5.29	5.55 ± 0.76	11.73 ± 5.97
0.325 ng/g	20.80 ± 4.16	5.98 ± 0.65	12.40 ± 5.54

4.2.5 *Corticosterone*

No significant differences were found among groups for ACTH response when controls and dose groups were compared (χ^2=10.08, df=5, p=0.0727). A significant difference was found among the four PCB dosed groups for ACTH response (χ^2=8.0327, df=3, p=0.0453). The highest dose group (0.325 ng/g) had a significantly lower response to ACTH stimulation. This data suggests that the 0.325 ng/g dose of PCB 126 was associated with a reduced stress response to the ACTH injection. This test may be a useful measure of contaminant burden.

Table 4.2.5.1. Mean ACTH response by dose, measured as the difference in corticosterone levels between T_0 and T_{30}.

Dose Group	ACTH response (ng/ml)
Control	112.80 ± 42.16
Vehicle Control	84.40 ± 37.86
0.100 ng/g	102.33 ± 29.50
0.175 ng/g	100.80 ± 30.22
0.250 ng/g	103.00 ± 59.22
0.325 ng/g	12.75 ± 18.34

4.2.6 *Immunotoxicology*

Controls were pooled for each test since control and vehicle control groups were not significantly different ($p>0.05$) for any variable except live thymocytes and the Jonckheere test requires only one control group.

PHA skin response test
There was a slight decline in skin test response with dose ($p=0.136$). The response at 0.1 ng/g was elevated (16%) above control with the higher doses all decreased 20% from the 0.1 ng/g dose. The three highest doses were 5-11% lower than pooled control. The PHA skin test response test is consistently suppressed 30-45% in herring gulls and Caspian terns from colonies on highly contaminated sites in the Great Lakes as compared to the least contaminated reference sites. The data from this study suggest that PCB 126 is playing a role in this suppression. Compared to wild birds, these chickens were only exposed to the contaminant during embryo development. Further exposure post-hatch through fish consumption may be driving the suppression to greater levels than that seen in the laboratory studies.

Antibody Response to Hemagglutination Assay
Five birds in the antibody response had titers of zero. According to notes taken at the time of immunization, they were successfully immunized. Due to this anomaly, the data for this endpoint were analyzed both with these endpoints and without them. The birds which had the zero titers were in the 0.1 and 0.325 ng/g dose group, 2 and 3 individuals respectively. The vehicle control, pooled control and 0.25 ng/g dose groups did not contain any zero titers.

Including zero titers: There was a decline in antibody response in all PCB exposed groups. Although the control and vehicle control mean titers were not significantly different ($p=0.17$), the vehicle control mean titer was 2.3 units (>4 fold) higher than the control. The mean titer for the 0.1 ng/g dose group was less than one half of the pooled control titer but titers were higher in the three highest dose groups. Considering total quantity of antibody, a decrease in one titer unit represents a 50% decrease in total antibody as the titer is on a $\log_2$ scale. Therefore, the mean total antibody of all PCB dosed groups except 0.25 ng/g were less than 50% of the pooled control. If antibody titers are compared only to vehicle controls, titers are close to 2 units lower (75% less) than control for all dose groups.

Discarding zero titers: Discarding the zero titers elevated the antibody response in the 0.1 and 0.325 ng/g groups. There was not a decreasing trend in antibody titer in this data set ($p=0.271$). However, all PCB dose groups except 0.25 ng/g still had less than 50% total antibody compared to controls.

Immune Organs
At two weeks of age, thymus index showed a significant decreasing trend with increasing dose ($p=0.0012$). Mean thymus index of birds in the 0.25 and .0325 ng/g groups were 24 and 22 % less than controls respectively. Thymic atrophy (decreased thymus index) in herring gull chicks and embryos has been associated with organochlorine exposure (unpublished data). Day 20 chicken embryos exposed to PCB 126 in the same manner as this study have thymic atrophy starting at about 0.128 ng/g. The apparent persistence of thymic atrophy to two weeks post-hatch

in this study shows the persistence of organ atrophy or delay in recovery from atrophy. Thymic atrophy may reduce the immune competence of an individual or delay the development of the immune system.

There was no decreasing trend in mean number of live thymocytes with dose (J=-0.27, J represents the Jonckheere statistic, negative Js are not significant). There was a significant difference between control and vehicle control birds (p=0.050), vehicle control birds having 56% less thymocytes than control birds. Sample sizes in these two groups were low, 4 and 6 birds per group respectively, which may have increased the chance for the large difference. Compared to pooled controls, the difference in mean number of thymocytes for each PCB exposed group in ascending order were: 24% decrease, 54% increase, 7% decrease, 30% increase. The lack of a trend in these results is surprising considering the decreasing trend in thymus index. In fact, compared to vehicle controls there appears to be an increasing trend in thymocyte number with dose. The increase in 0.175 and 0.325 dose groups are driven by one extreme in each case. Discarding these extremes and an extreme from the 0.1 ng/g group results in a less negative J value (-0.058).

Since the birds were euthanized over two days, some birds were euthanized at 13 days old and the others at 14 days old. At about two weeks of age, the last major wave of mature thymocytes exits the thymus for the periphery. In the event that the difference in age of birds at time of thymocyte counts may explain the lack of monotonic trend in the data, the counts were split up by age of bird. The data between the two days is quite different, with an apparent decline in thymocyte count at the two highest doses at day 13, but an apparent increase at these doses at day 14.

Thymocyte density (number of live thymocytes in left thymus divided by organ mass) was calculated using both day 13 and 14 data. There was a slight increasing dose response.

Bursa index significantly decreased with dose (p=0.0001) at two weeks of age. The 0.325 ng/g dose group was significantly decreased (22%) from control (p>0.05). The mean live bursal cell count in the lowest dose group (0.100 ng/g) was increased from controls (36%) and compared to it, each higher dose group declined in mean bursal cell count. The highest dose group's count was 25% less than pooled control. The live bursal cell data was split into day 13 and 14 as with the thymocyte data. The controls have a similar number of bursal cells on both days (unlike the thymocyte data). At day 13, there is a clear decline in bursal cell number at the highest dose. At day 14, there is a dramatic increase in live cell number in all PCB exposed groups but there is still a clear descending trend in cell number within PCB exposed birds. This data could suggest a delay in bursal cell development, i.e. delayed proliferation or delayed influx of precursors compared to control birds.

Bursal cell density was calculated using both day 13 and 14 and shows a decreasing trend within the PCB exposed birds. Bursal atrophy occurs in chicken embryos exposed to similar PCB 126 doses as in this study. The apparent persistence of bursal atrophy to two weeks post-hatch in this study shows the persistence of organ atrophy or delay in recovery from atrophy. Bursal atrophy may reduce the immune competence of an individual or delay the development of the immune system.

The lack of consistency between organ mass and cell counts, especially in thymocytes, is surprising. It is possible that the dosing regime, i.e. delay in incubation of injected eggs, could explain some of these differences. Since the eggs were dosed over two days and set on the third day, resulting in 50% of the eggs sitting an additional 24 hours before incubation, the dynamics of exposure may be different between the two groups.

There was not a significant declining trend in either thymic or bursa index at the 5-week necropsies (35 days of age). This suggests a recovery from thymic atrophy at the two highest doses. There appears to be a full recovery from bursal atrophy at the 0.175 and 0.25 ng/g doses but not at the 0.325 ng/g dose. At day 14, the bursal index in the 0.325 ng/g group was 22% less than controls, at day 35 the thymic index of this group was 10% less than controls.

4.2.7 *Cytochrome P450/Phase 2 Enzymes*

The results show that EROD activity for CYP1A was still significantly induced (50 pmol/mg/min) in the animals with the two highest PCB-doses. In general, CYP1A induction peaks in 3-5 days after a single dose. The results shown here, where there is still significant induction after two weeks, may indicate that there is continuous release of PCB from the fat resources in the developing chickens.

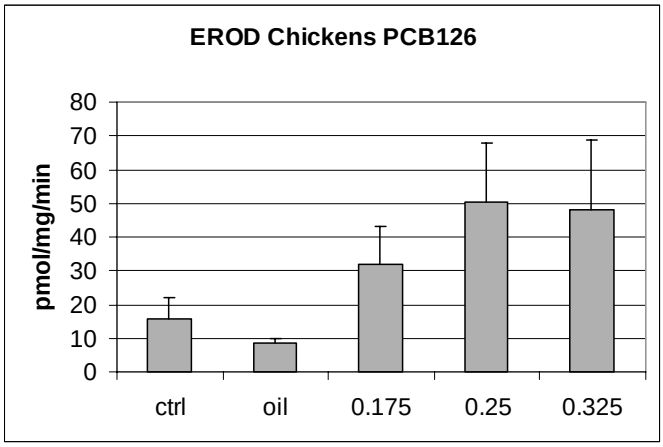

Figure 4.2.7.1. EROD activity in 2-week old chickens.

The GST activity shows the same pattern as the EROD activity, the highest doses display significantly higher GST activity (900 nmol CDNB/mg/min) than the control, as seen in Figure 4.2.7.2.

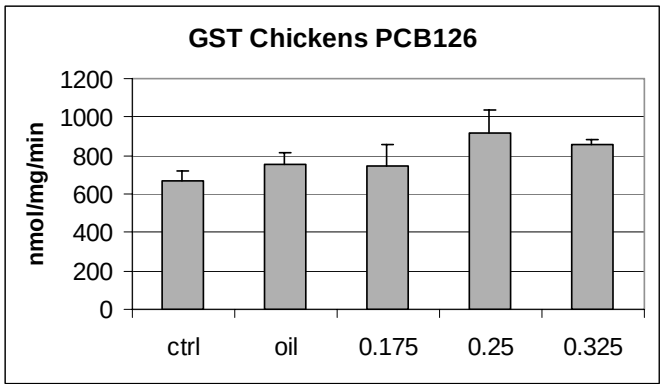

Figure 4.2.7.2. GST activity in 2-week old chickens.

For the UGT and SULT activities no significant differences were detected among the treatment groups (p=0.14 and 0.69 resp.). However, there seems to be a trend of increasing UGT activity (35 pmol/mg/min) and decreasing SULT activity (60 pmol/mg/min) in the higher doses compared to the controls. High individual variability masks the trend, as expressed in the high standard deviations. It appears though, that the enzymes behave as expected: SULT is normally not inducible, and inhibition of the constitutively expressed enzyme results in a lower overall activity. UGT on the other hand is inducible, and higher activities in the higher doses may indicate an induced amount of active enzyme, which compensates for the loss of activity in SULT and constitutively expressed UGT. Further analysis of the actual enzyme concentrations, using gel electrophoresis followed by immunodetection in Western blots, is under way.

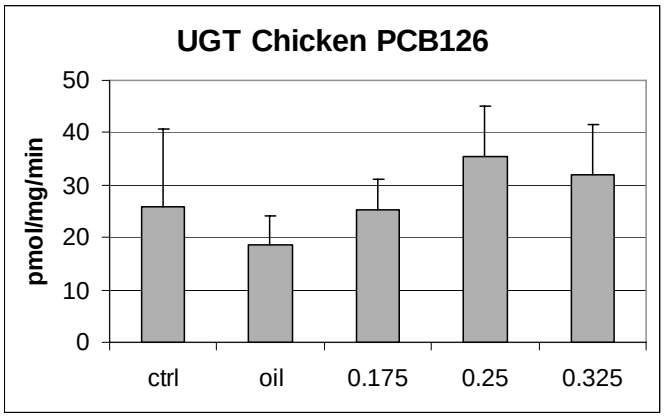

Figure 4.2.7.3. UGT activity in 2-week old chickens.

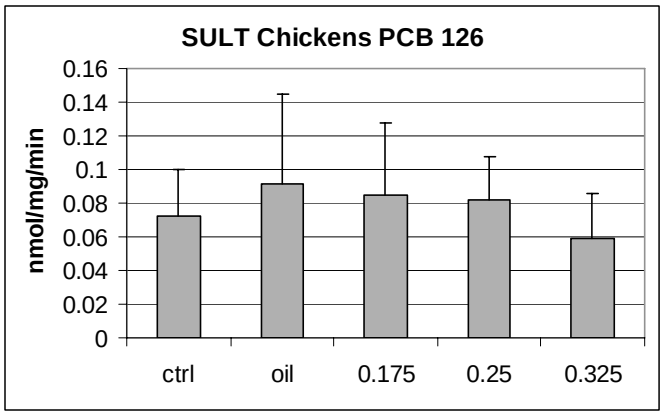

Figure 4.2.7.4. SULT activity in 2-week old chickens.

4.2.8 *Intestinal Bacteria*

Microbial Identification
Two-week samples:
All of the samples contained the gram-negative bacteria *Escherichia coli.* The other predominant gram-negative bacterial type was *Klebsiella pneumoniae* and the predominant gram-positive bacterial type was *Enterococcus gallinarum.* A few other colony types were identified randomly among the dose groups, including the Enterobacter species *E.cloacae*, *E.sakazakii*, and *E.taylorae*, and the Enterococcus species *E.faecium*, *E.faecalis*, and *E.casseliflavus*. These colonies looked similar to the predominant types and were analyzed unintentionally. A significant difference was noted among the dose groups for presence or absence of a second gram-negative bacteria (p=0.0189). 87.5% and 100% of control and vehicle control samples contained a second gram-negative type, respectively, while only 77.8% (0.1 ng/g), 20% (0.175 ng/g), 60% (0.25 ng/g), and 50% (0.325 ng/g) of the dose groups contained a second gram-negative type, as shown in Figure 4.2.8.1.

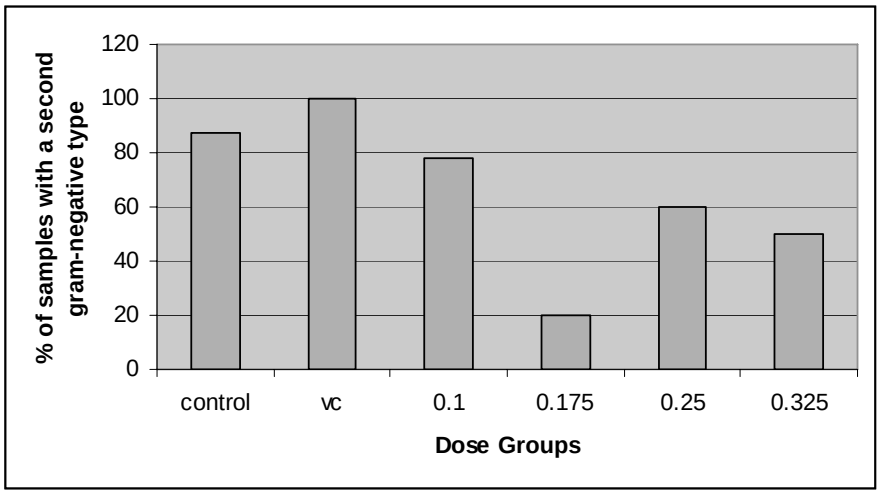

Figure 4.2.8.1. Percent of samples per dose group containing a second gram-negative bacterial type.

A significant difference was also noted in the distribution of gram-positive bacterial types among the dose groups (p=0.0746). *E.gallinarum* was the predominant bacterial type found, comprising 90-100% of the colonies identified in the vehicle control and each of the four dose groups. However, the control group contained only 37.5% *E.gallinarum*, as shown in Figure 4.2.8.2. When the control group was tested against the other doses, a significant difference was evident (p=0.0001).

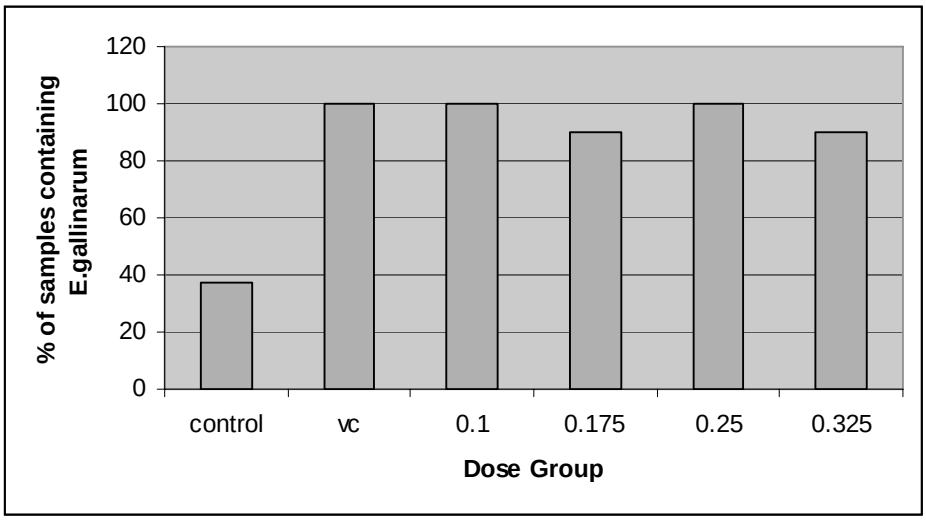

Figure 4.2.8.2. Percent of samples per dose group containing *E.gallinarum*.

Five-week samples:
As in the two-week birds, all samples contained *E.coli*. No significant difference was noted among the dose groups for presence or absence of a second gram-negative bacteria (p=0.5724), nor were there significant differences in the type of gram-positive bacteria present (p=0.3897).

Results obtained for microbial identification may show dose-related differences in bacterial types present. The presence or absence of a second gram-negative bacteria was the most significant difference. The presence of a second bacteria was more likely in the control and vehicle control birds than in the PCB 126 groups. These results were not clearly dose related though, with the lowest incidence of a second bacteria in the second dose concentration (0.175 ng/g).

As for the differences in types of bacteria found, there was a significant difference in the gram-positive bacteria identified. The control group contained a relatively even mixture of four bacterial types, while the other groups contained 90-100% *E.gallinarum*. However, because only three bacteria were consistently tested, it is not clear whether the results are dose related or simply due to random testing of similar looking bacteria. Before testing began, it was determined to analyze only the three predominant bacterial types, due to monetary reasons and time constraints. Only one gram-positive colony type was tested for each bird. The different bacterial types of the controls were identified unintentionally, because they looked similar to the predominant type. The majority of the samples did only contain one gram-positive type, but some of the samples had more. These additional types were not tested. In the next phase of the project, it is recommended that each bacterial type observed per sample be tested. This additional

testing should not require much more time or money, if the numbers of bacterial types present are similar to this study. Testing each colony type would give a more accurate picture of the differences in bacterial types among the dose groups.

Antibiotic Susceptibility Profiles

Gram-negative/2 week:

Significant differences were noted among dose groups for susceptibility of *E.coli* to several of the antibiotics, including Cephalothin (p=<0.0001), Ampicillin/Sulbactam (p=<0.0001), Ampicillin (p=<0.0001), Piperacillin (p=<0.0001), Gentamicin (p=0.0003), and Tobramycin (p=0.0070). For each of these antibiotics, there is a significant difference between the susceptibility of the control birds and that of the other groups.

Table 4.2.8.1. Percent of susceptible *E.coli* per dose group.

Group	Cephalothin	Amp/Sulb	Ampicillin	Piperacillin	Gentamicin	Tobramycin
Control	100%	100%	100%	100%	75%	75%
Vehicle Control	25%	50%	50%	50%	25%	25%
0.1 ng/g	11.11%	11.11%	11.11%	11.11%	11.11%	22.22%
0.175 ng/g	0%	0%	0%	0%	0%	0%
0.25 ng/g	40%	50%	40%	40%	10%	20%
0.325 ng/g	0%	0%	0%	0%	0%	10%

The vehicle control group shows difference from the controls. The small sample size for the vehicle control group (n=4) may be a factor in this difference. Fisher's exact test was used to eliminate the sample size problem. These results showed no significant differences in control groups except with Cephalothin (p=0.182). There is also no clear trend in susceptibility with dose concentration. Figure 4.2.8.3 shows the percent susceptibility of *E.coli* per dose group to each antibiotic.

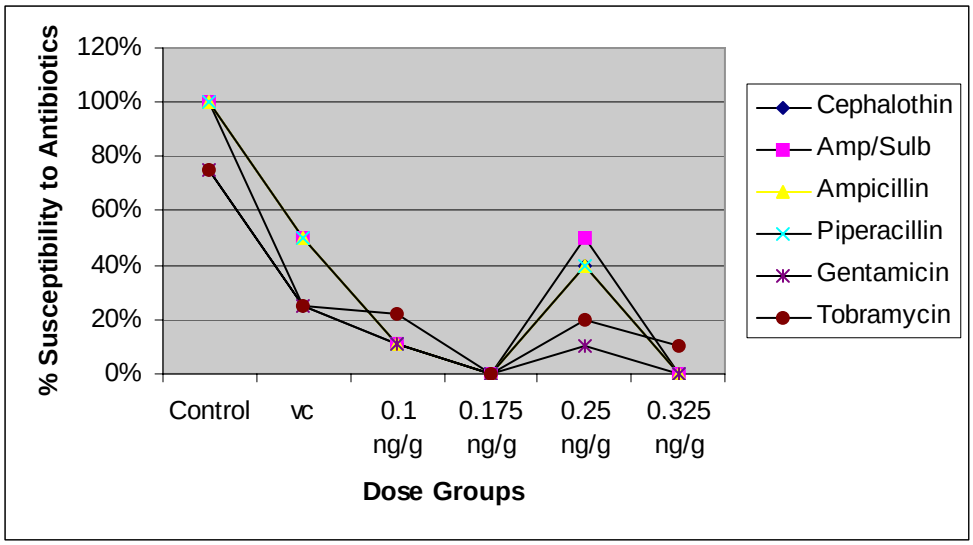

Figure 4.2.8.3. Percent of susceptible *E.coli* per dose group.

The second predominant gram-negative bacteria, *K. pneumoniae*, showed no significant difference in susceptibility among dose groups for all antibiotics except Ampicillin/Sulbactam. For this antibiotic there was a significant difference in susceptibility between the highest dose concentration and the other doses/controls (p=0.0111). 100% of the bacteria in control, vehicle control, and the lowest three dose groups were susceptible, whereas only 40% of the highest dose group were susceptible.

The other gram-negative bacteria occurred too randomly to compare susceptibility among the doses.

Gram-negative/5 week:
No significant differences were noted among the dose groups for *E.coli* susceptibility to any of the antibiotics used. A significant difference was noted between the highest dose concentration and the remaining groups for *K.pneumoniae* susceptibility to Colistin and Ampicillin/Sulbactam (p=0.0261 for both). With both of these antibiotics, the bacteria in the control, vehicle control, and lower dose groups were 100% susceptible. In the highest dose group, only 60% of the bacteria were susceptible. No other differences were noted for *K.pnuemoniae*.

Gram-positive/2 week:
Significant differences were noted among the dose groups for *E.gallinarum* susceptibility to Gentamicin, Ciprofloxacin, and Norfloxacin (p=<0.0001 for each). However, these differences were not dose related. For each of these antibiotics, the control and the two high doses had similar susceptibility and differed from the vehicle control and two lower doses, as illustrated in Figure 4.2.8.4.

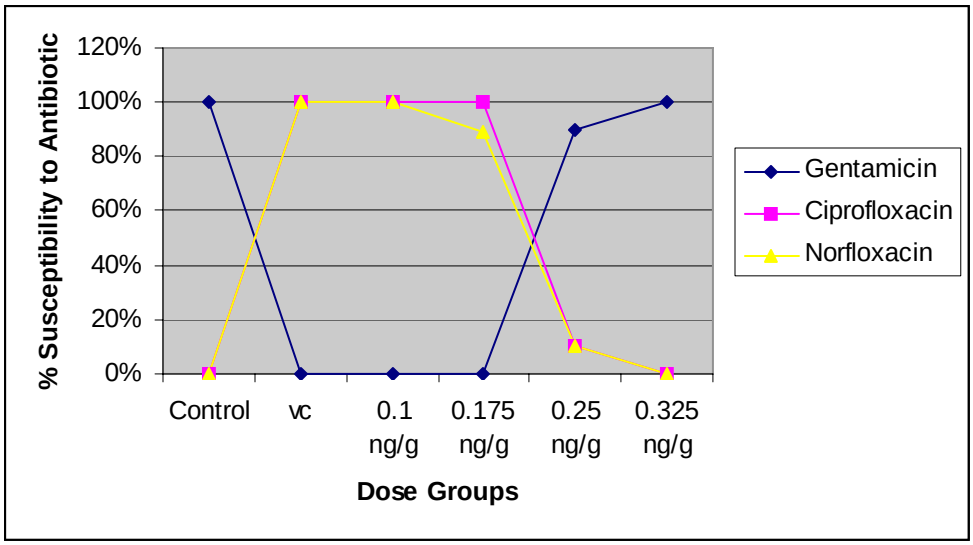

Figure 4.2.8.4. Percent of susceptible *E.gallinarum* per dose group.

The other bacteria identified did not occur in large enough numbers to compare susceptibility among doses.

Gram-positive/5 week:
No significant differences in susceptibility were noted among the dose groups for any bacteria.

If the differences observed between doses are related to the PCB 126, then some of these results do moderately support my hypothesis. For instance, the antibiotic susceptibility of *E.coli* shows differences between the control and the dose groups. All of the control birds were 100% susceptible to the antibiotics, while the dose groups were significantly less susceptible. These results may show that the bacteria in the dosed birds were more resistant strains than in the controls. However, the vehicle control birds also showed less susceptibility and there was not a clear concentration related response. There were also significant differences in *E.gallinarum* susceptibility but they were not dose-related.

One factor to consider is that the chicks were placed in the brooder levels according to dose group. This was done in an effort to lower handling time and stress levels when taking birds out to be weighed and tested. The slight difference in their environment could have been a factor in the differences observed between dose groups for both the microbes identified and the susceptibility profiles. For the next dosing experiment, it is recommended that the birds be randomized in the brooder, in order to rule out this variable. Since the birds will have color-coded wing tags, this should not cause much more difficulty in sorting birds for testing.

Another interesting observation is that while the two-week birds contained some significant differences, the five-week birds did not. If the PCB 126 was causing some effect on the bacteria present, this effect did not continue to five weeks of age. This may suggest that the contaminants were metabolized and not causing further effects at the older age.

SECTION 5.0

FUTURE STUDIES

5.1 DOUBLE-CRESTED CORMORANT EXTRACT DOSING

5.1.1 *Failed Experiment*

Double-crested cormorant extract dosing took place in March of 2002. Eggs were dosed with four concentrations of the extract as provided by Dr. Donald Tillitt, USGS-BRD. The extract was delivered in a sunflower oil carrier and all procedures were identical to the PCB 126 dosing. Eggs hatched on 4-5 April 2002. Mortality was extremely high, as seen in Table 1.

Table 5.1.1.1. Eggs Dosed and Hatched – Extract Dosing

Treatment	**# Eggs Dosed**	**# Eggs Hatched**	**Percent Mortality***
Control	46	27	41.3%
Vehicle Control (sunflower oil)	36	18	50%
G1 (0.0625 egg-EQ)	36	12	66.7%
G2 (0.125 egg-EQ)	36	5	86.1%
G3 (0.250 egg-EQ)	59	5	91.5%
G4 (0.50 egg-EQ)	81	5	93.8%
Total	294	68	76.9%

* Percent mortality includes all eggs that did not hatch, including infertiles.

Eggs that failed to hatch were examined for stage of death on 12 April. Deformities were noted in many of the eggs during examination as shown in Table 2. Types of deformities included edema, gastroschisis, crossbills, clubfoot, and other beak and foot deformities. Small body size was also common. These deformities were noted with each concentration of extract, as well as a few in the vehicle control group. The control eggs contained no observable deformities.

Table 5.1.1.2. Number of observable deformities per dose group.

Dose Group	Number of Deformities	% Deformities
Control	0	0
Vehicle Control	6	16.7
0.0625 egg-EQ	5	13.9
0.125 egg-EQ	9	25
0.250 egg-EQ	24	40.7

0.50 egg-EQ	22	27.2
Total	66	22.4

Due to the small sample sizes in the high dose groups, it was decided to euthanize all birds and repeat the experiment at a later time. It is not clear what caused the high mortality in this experiment. Previous experiments done with the same extract resulted in much lower mortality (Powell et al 1996). Since the control groups had low hatchability as well as the dosed groups, it is thought that the eggs may have been less viable than in the previous experiment.

5.1.2 *Fall 2002 Cormorant Extract Dosing*

A second trial with the cormorant extract is scheduled to begin in September of 2002. Mortality data from the first experiment is being taken into consideration in planning the next dosing. Concentrations of extract may be lowered and the number of eggs dosed will be increased. Endpoints for this experiment will be the same as in the PCB 126 experiment. Final necropsies should take place in November of 2002 with analysis following. In the event that not enough birds hatch to do both the 2-week and 5-week groups, a second dosing will take place in November. In this case, the first group of birds would all be taken out to 5-weeks of age and the second dosing would provide birds for the 2-week study. The second dosing will be cancelled if a sufficient number of birds hatch in October. The project schedule is outlined below.

CORMORANT EXTRACT SCHEDULE

September 10-12	Dose and set eggs
October 3	Hatch
October 14-15	PHA skin test
October 16-18	2-week necropsy
October 25	SRBC injections
October 31	Blood collection for SRBC
November 1,4	ACTH test
November 6-8	5-week necropsy
November 12-14	2^{nd} dosing
December 5	Hatch
December 18-20	2-week necropsy

5.1.3 Additional Biomarkers

We are awaiting results for vitellogenin and estrogen levels from both the bald eagle field trials and the controlled chicken experiment. These results, in additon to the cormorant extract study results will be included in the final report.

SECTION 6.0

ACKNOWLEDGEMENTS

Special thanks and appreciation are extended to the following people and universities for their assistance in conducting this study and/or completing this report:

USGS-BRD
Dr. Donald Tillitt

Clemson University
Kathy Bryant
Ann Miller
Furman Cantrell
Godley Snell Research Center Staff
Wildlife Toxicology Lab Students

Field Crew
John B. Holt, Jr.
Teryl Grubb
Joseph Papp
Sergej Postupalsky

Voyageurs National Park
Lee Grim
Jim Shaberl

SECTION 7.0

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